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Short Communication

Neutralizing Antibodies against Epidermal Growth Factor and ErbB-2/*neu* Receptor Tyrosine Kinases Down-Regulate Vascular Endothelial Growth Factor Production by Tumor Cells *in Vitro* and *in Vivo*

Angiogenic Implications for Signal Transduction Therapy of Solid Tumors

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The overexpression in tumor cells of (proto)-oncogenic receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) or ErbB2/*neu* (also known as HER-2) is generally thought to contribute to the development of solid tumors primarily through their effects on promoting uncontrolled cell proliferation. However, agents that antagonize the function of the protein products encoded by these (proto)-oncogenes are known to behave *in vivo* in a cytotoxic-like manner. This implies that such oncogenes may regulate critical cell survival functions, including angiogenesis. The latter could occur as a consequence of regulation of relevant growth factors by such oncogenes. We therefore sought to determine whether EGFR or ErbB2/*neu* may contribute to tumor angiogenesis by examining their effects on the expression of vascular endothelial cell growth factor (VEGF)/vascular permeability factor (VPF), one of the most important of all known inducers of tumor angiogenesis. We found that *in vitro* treatment of EGFR-positive A431 human epidermoid carcinoma cells, which are known to be heavily dependent on VEGF/VPF *in vivo* as an angiogenesis growth factor, with the C225 anti-EGFR neutralizing antibody caused a dose-dependent inhibition of VEGF protein expression. Prominent suppression of VEGF/VPF expression *in vivo*, as well as a significant reduction in tumor blood vessel counts, were also observed in established A431 tumors shortly after injection of the antibody as few as four times into nude mice. Transformation of NIH 3T3 fibroblasts with mutant ErbB2/*neu*, another EGFR-like oncogenic tyrosine kinase, resulted in a significant induction of VEGF/VPF, and the magnitude of this effect was further elevated by hypoxia. Moreover, treatment of ErbB2/*neu*-positive SKBR-3 human breast cancer cells *in vitro* with a specific neutralizing anti-ErbB2/*neu* monoclonal antibody (4D5) resulted in a dose-dependent reduction of VEGF/VPF protein expression. Taken together, the results suggest that oncogenic properties of EGFR and ErbB2/*neu* may, at least in part, be mediated by stimulation of tumor angiogenesis by up-regulating potent angiogenesis growth factors such as VEGF/VPF. These genetic changes may cooperate with epigenetic/environmental effects such as hypoxia to maximally stimulate VEGF/VPF expression. Therapeutic disruption of EGFR or ErbB2/*neu* protein function *in vivo* may

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therefore result in partial suppression of angiogenesis, a feature that could enhance the therapeutic index of such agents *in vivo* and endow them with anti-tumor effects, the magnitude of which may be out of proportion with their observed cytostatic effects in monolayer tissue culture. ***(Am J Pathol 1997, 151:1523–1530)***

One of the major cellular changes that accompanies tumor development and progression is overexpression of proto-oncogenic protein receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) or the ErbB2/*neu* (also known as HER-2).[1] Given their increased expression in many types of solid tumors, and location at the external cell surface, there has been considerable interest in developing and utilizing agents that block in a relatively selected way the signaling function of these receptor tyrosine kinases.[2,3] These agents include specific neutralizing monoclonal antibodies, such as 4D5 and C225, which block the human ErbB2/*neu* and EGFRs, respectively. Both are now being evaluated in early-phase clinical trials, either alone or in combination with various cytotoxic anti-cancer chemotherapeutic drugs with which they may synergize.[4]

As with most other agents that act as inhibitors of signal transduction, such blocking antibodies are generally viewed as cytostatic drugs. This is based on the observation that they usually appear to lack any obvious cytotoxic properties when tested against relevant tumor cell targets grown in monolayer tissue culture. Despite this, there are situations in which these antibodies or agents appear to exert therapeutic effects against established human solid tumors in preclinical animal models, the magnitude of which are strongly suggestive of the involvement of a cytotoxic, or cytotoxic-like, effect. A similar discrepancy has been noted with respect to protein farnesyltransferase inhibitors of mutant RAS oncoproteins.[5] For example, the maximal anti-tumor effect of the C225 monoclonal anti-EGFR antibody against A431 human epidermoid carcinoma cells in culture is approximately 35% growth inhibition, with no cytotoxicity.[6] Nevertheless, injections of this antibody into nude mice harboring established A431 xenografts can result in total regression of the tumors within a relatively short period of time.[6] A similar *in vitro/in vivo* therapeutic discrepancy has been noted with the 4D5 anti-ErbB2/*neu* antibody.[7] The extent of these *in vivo* therapeutic effects are all the more surprising given the usual physiological and pharmacokinetic problems, such as antibody delivery into solid tumors, that exist *in vivo* but not *in vitro* to limit the therapeutic potential of many anti-cancer agents.[8] These observations suggest that the antibodies may acquire anti-tumor mechanisms of action *in vivo* not normally detected in monolayer tissue culture, such as, for example, antibody-dependent cell-mediated cytotoxicity. Perhaps an even more appealing possibility is inhibition of tumor angiogenesis.

The notion that signal transduction inhibitor drugs may function as anti-angiogenic agents, which we first put forward in the context of the effects of protein farnesyltransferase inhibitors of mutant RAS oncoproteins,[9] is based on the hypothesis that oncogene/proto-oncogene-mediated signal transduction pathways may up-regulate the expression of one or more growth factors that function as stimulators of angiogenesis.[9,10] One such factor is vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), which is currently regarded as the major angiogenesis stimulator for most types of human cancers.[11] Indeed, of the many known inducers of VEGF/VPF, two of the most potent are epidermal growth factor (EGF) and transforming growth factor (TGF)-α[12,13] which are ligands for the EGFR. Hence, anti-EGFR antibodies might be expected to suppress VEGF/VPF expression both *in vitro* and, possibly, *in vivo*. If so, this could result in an anti-angiogenic effect that might even lead to tumor regression, given that even partial VEGF withdrawal can lead to destruction of newly formed immature vessels, such as those found in neonatal retinas[14] and solid tumors,[15] in addition to inhibiting the development of new blood vessels.

The purpose of the present study was to evaluate whether EGFR or ErbB2 induces or up-regulates VEGF/VPF expression and, if so, whether pharmacological or genetic blockade of either EGFR or ErbB2/*neu* receptor kinases can lead to a suppression of VEGF/VPF production, both *in vitro* and *in vivo*, using appropriate receptor-bearing murine or human target tumor cells. The pharmacological approach was undertaken using monoclonal neutralizing antibodies specific for either receptor kinase. Our decision to focus on VEGF/VPF as an overall surrogate marker of angiogenesis was based on several considerations, namely, 1) its ubiquity as a tumor angiogenesis factor,[11] 2) that relatively small reductions (two- to threefold) in VEGF/VPF can lead to unexpectedly profound suppressions of developmental[16] and tumor angiogenesis,[17] and 3) that it is known to be a major angiogenesis growth factor for the A431 human squamous carcinoma,[18] which was the tumor selected for our *in vivo* therapy studies reported here. The results we obtained showed that these agents can indeed suppress tumor VEGF/VPF expression, both in cell culture and *in vivo*, and therefore raise the possibility that these agents may have an anti-angiogenic component as part of their mode of anti-tumor action *in vivo*.

Materials and Methods

Cell Lines and Culture Conditions

The human epidermoid carcinoma cell line, A431, was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained as a monolayer culture in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY). This cell line is known to overexpress EGFR. The human breast adenocarcinoma cell line SKBR-3, which overexpresses ErbB2/*neu*, came from two independent sources, namely, the ATCC and as a gift from Dr. Peter Taylor (Instituto Venezolano de Investigaciones Científicas IVIC, Caracas, Venezuela). These cells

were maintained in DMEM/F12 medium supplemented with 10% FBS (Gibco-BRL). B104.1.1. cells are transformed NIH 3T3 fibroblasts generated by transfection with a mutant *neu* (the rat homologue of the human ErbB-2) oncogene originally identified in a rat neuro(glio-)blastoma.[19] This cell line and its parental NIH 3T3 counterpart were cultured in DMEM/F12 supplemented with 10% FBS.

Antibodies

The neutralizing anti-EGFR monoclonal antibody C225, originally described by Kawamoto et al,[20] was produced by ImClone Systems (New York, NY). Murine monoclonal antibody against the extracellular domain of the human ErbB2/*neu* receptor 4D5[3] and the anti-VEGF antibody A4.6.1 were produced by Genentech (South San Francisco, CA).

Measurement of Human and Mouse VEGF Protein Levels in Conditioned Medium (ELISA)

Commercially available human or mouse VEGF ELISA kits (R&D Systems, Minneapolis, MN) were used to quantitate the levels of VEGF in conditioned medium obtained from A431 and SKBR-3 or NIH 3T3 and B104.1.1. cells, respectively, according to the manufacturer's instructions. Briefly, cells were plated at a density of 10^5 (A431 and SKBR-3) or 75×10^3 (NIH 3T3 and B104.1.1.) cells/0.5 ml/well in a 24-well plate and allowed to reach near confluency, at which point the growth medium was replaced with fresh assay medium containing C225, 4D5 monoclonal antibodies, or nonspecific IgG (as a control), FBS and, where indicated, 100 μmol/L $CoCl_2$. Conditioned medium was collected after 24, 36, or 48 hours, cellular debris removed by centrifugation, and medium kept at −70°C until VEGF quantitation was undertaken. Cell number was determined immediately after medium recovery using a Coulter Counter ZM (Coulter Electronics, Luton, UK). Cobalt chloride was used to mimic hypoxic conditions in cell culture.[21]

Northern Blotting

Approximately 10^8 cells were used for the extraction of polyadenylated mRNA by a standard SDS-oligodeoxythymidylic acid method. The RNA was resolved on 1% agarose gel containing 6.6 mol/L formaldehyde, transferred to Zeta Probe (Bio-Rad, Hercules, CA) membrane, and hybridized at 65°C with a ^{32}P-labeled cDNA probe containing 200-bp human VEGF sequence common for all four known VEGF/VPF isoforms (a gift from Dr. Brygida Berse and Dr. Harold Dvorak, Beth Israel Hospital, Boston, MA). The membranes were autoradiographed after the transfer, and the intensity of the 3.7- and 4.5-kb VEGF/VPF signal was evaluated.

Evaluation of the in Vivo Anti-Tumor Activity of the C225 Anti-EGFR Antibody

A431 cells were cultured to semiconfluency in their appropriate growth medium. The cultures were harvested by brief trypsin/EDTA (GIBCO-BRL) treatment, washed in serum-free medium, resuspended at the appropriate density in PBS, and then inoculated subcutaneously (s.c.) at a density of 10^6/0.2 ml into athymic 8-week-old nu/nu BALB/c mice. After 15 days (when tumors reached a size of 200 to 300 mm^3), treatment was initiated by injecting intraperitoneally 1 mg. of C225 monoclonal antibody per mouse every other day, for a total of four injections. Control mice were injected with PBS. Tumors were measured periodically, and tumor volume (mm^3) was calculated by using the standard formula $a^2 \times b/2$, where a is the width and b is the length of the horizontal tumor perimeter. The experiment was terminated after 1 week, when tumors were removed and immediately frozen in ornithine carbamyl transferase compound (Tissue-Tek) or formalin fixed for immunohistochemistry. Similarly, as a control *in vivo* experiment, mice bearing established A431 tumors were treated with two injections, 3 days apart, of anti-VEGF A4.6.1 antibody (300 μg/mouse or 0.2 ml of PBS), and tumor tissue was processed for immunohistochemistry.

Immunohistochemistry and Blood Vessels

The formalin-fixed, paraffin-embedded specimens of individual control or antibody-treated tumors were sectioned and processed for standard immunohistochemical staining. Anti-VEGF rabbit polyclonal antibody A-20 (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:200 dilution in combination with a proper secondary antibody from the Histostain-SP kit (Zymed Laboratories, San Francisco, CA) and 3-amino-9-ethyl carbazole (AEC) chromogen to reveal antigen as a red signal. Anti-Ki67 rabbit polyclonal antibody NCLki67p (Novocastra Laboratories, New Castle, UK) was used at a 1:1000 dilution. The color reaction was developed by using an anti-rabbit secondary antibody, the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine tetrahydrochloride (DAB, Pierce, Rockford, IL) as a chromogen to obtain brown coloration. Blood vessel staining was performed on unfixed frozen sections by endothelial cell labeling with GSI lectin from *Griffonia simplicifolia* as previously described.[22]

Statistical Analysis

The Mann-Whitney nonparametric test for unpaired data was used to evaluate the immunohistochemistry results. All P values represent two-sided tests of statistical significance. The analyses were performed using the GraphPAD InStat program version 1.14 (GraphPAD Software, Inc., San Diego, CA).

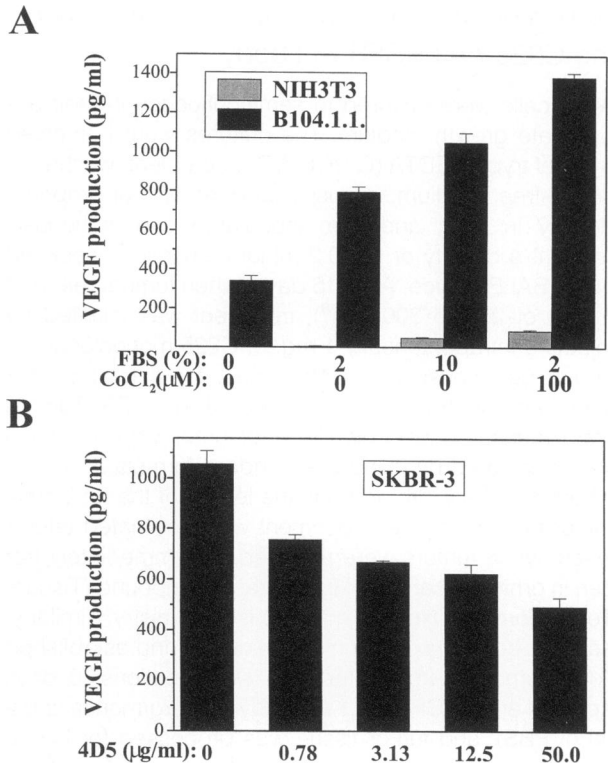

Figure 1. VEGF production by cells transformed with ErbB2/*neu* oncogene. **A:** Up-regulation of VEGF immunoreactivity in conditioned medium of ErbB2/*neu*-transformed NIH 3T3 cells (B104.1.1). VEGF production is stimulated by serum and hypoxia ($CoCl_2$) treatment in both B104.1.1 and control NIH 3T3 cells, but maximal levels in the case of the former cells are up to 100-fold higher. **B:** Dose-dependent down-regulation of VEGF production by SKBR-3 human breast carcinoma cells after 48 hours of treatment with 4D5 (anti-ErbB2/*neu*) antibody. Error bars, SD.

Results

Up-Regulation of VEGF Production in Fibroblasts Transformed with ErbB2/neu Oncogene

Transformation of NIH 3T3 fibroblasts with a rat oncogenic mutant of the ErbB2/*neu* receptor results in acquisition of a tumorigenic phenotype[23] for which expression of angiogenic properties is presumably an absolute requirement.[24] Therefore we decided to examine whether expression of VEGF, a potent angiogenic growth factor frequently regulated by oncogenic proteins such as mutant *ras*,[9,25–28] is also up-regulated in the case of fibroblastic cell line B104.1.1 transformed with ErbB2/*neu* oncogene.[19,29] Figure 1A shows the comparative analysis of VEGF protein production by B104.1.1 cells and their nontransformed parental NIH 3T3 cells under different culture conditions. In confirmation of the results of Grugel et al,[25] VEGF secretion into conditioned media by NIH 3T3 cells was virtually undetectable in the absence of serum. In striking contrast, B104.1.1 cells produced an abundance of this angiogenic growth factor. Exposure of parental NIH 3T3 cells to serum (2 to 10%) and hypoxia (100 μmol/L $CoCl_2$) resulted in secretion of measurable amounts of VEGF; however, the corresponding conditioned media of B104.1.1 cells contained up to 100-fold more immunodetectable VEGF. Thus, although hypoxic conditions, as expected, can induce VEGF in nontransformed cells, the magnitude of this effect is remarkably enhanced in the same cells when they contain an activated oncogene. This observation reinforces the notion that genetic and epigenetic factors cooperate to bring about an angiogenic switch during malignant transformation and tumor progression.[26,27,30]

*Down-Regulation of VEGF Production in SKBR-3 Human Breast Cancer Cells upon Treatment with 4D5 (Anti-ErbB2/*neu*) Antibody*

The aforementioned results suggest that through pharmacological blockade of the ErbB2/*neu* protein it should be possible to suppress VEGF/VPF expression. We decided to test this hypothesis using mouse neutralizing monoclonal anti-human ErbB2/*neu* antibodies and relevant human tumor cells. We could not use the *neu*-transfected variants of NIH 3T3 cells for such experiments as the antibody does not react against rodent *neu*. In human breast cancer cells, endogenous overexpression of the ErbB2/*neu* oncogene is frequently associated with tumor progression, and the neutralizing anti-ErbB2/*neu* antibody known as 4D5 (a specific monoclonal antibody against the human but not rat extracellular domain of ErbB2/*neu*) has been shown to possess significant antitumor properties *in vivo* in preclinical models.[7] We therefore employed SKBR-3 cells to assess whether treatment with this antibody is able to suppress VEGF production in the case of a natural, human, ErbB2/*neu*-transformed breast cancer cell line. This indeed appears to be the case as shown in Figure 1B. Although untreated SKBR3 cells were found to secrete appreciable quantities of VEGF (approximately 1000 pg/ml/10^5 cells) into the conditioned media, increasing concentrations of 4D5 antibody brought about a dose-dependent decrease in VEGF production, which reached 50% at the concentration of 50 μg/ml antibody, indicating that indeed ErbB2/*neu* activity can be at least one significant factor regulating VEGF/VPF expression in these cells.

Down-Regulation of VEGF Production in A431 Cells by Anti-EGFR Antibody C225 in Vitro

The tumorigenic transformed phenotype of A431 human epidermoid carcinoma cells is thought to be dependent, at least in part, on overexpression of EGFR. As monoclonal antibody C225, directed against the EGFR, has been shown to inhibit growth of established A431 tumors *in vivo*, we hypothesized that down-regulation of VEGF and ultimately tumor angiogenesis itself might be a contributing factor in causing such an anti-tumor effect. As a first step toward testing this hypothesis we first treated A431 cells *in vitro* with increasing concentrations of the C225 antibody and measured secretion of VEGF into conditioned media as well as expression of VEGF mRNA. Figure 2A shows that VEGF production was inhibited by the antibody treatment in a dose-dependent manner.

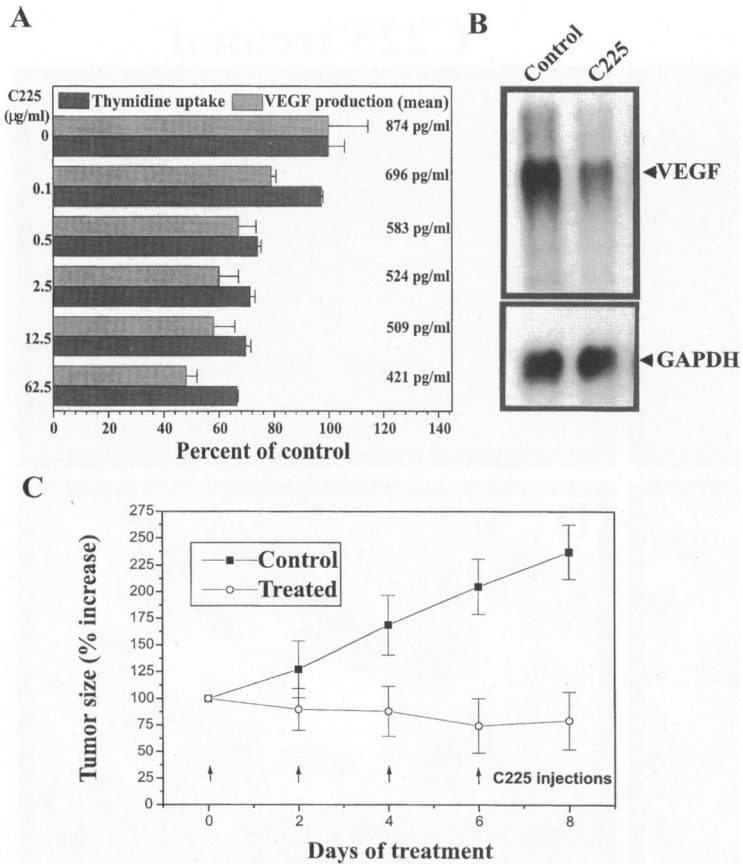

Figure 2. The effect of treatment with C225 anti-EGFR antibody on growth of A431 tumor cells *in vitro* and *in vivo* and their VEGF production. **A:** Dose-dependent inhibitory effect of C225 treatment on VEGF production and growth of A431 cells *in vitro*. **B:** Down-regulation of VEGF mRNA expression in A431 cells treated for 24 hours with 1 μg/ml C225 antibody. **Bottom panel:** loading control (GAPDH). **C:** Growth-inhibitory effect of C225 antibody on *in vivo* growth of established A431 tumors in nude mice after transient (1-week) treatment (time 0 corresponds to day 15 of tumor growth; error bars, SD; n ≥ 6 mice).

Although control A431 cells produced significant quantities of VEGF (800 to 900 pg/ml/10^5 cells), the amount of this cytokine produced by their counterparts treated for 24 hours with 62.5 μg/ml C225 antibody was approximately 50% lower (400 pg/ml/10^5 cells). Corresponding measurements of [^{3}H]thymidine incorporation indicated that the suppressive effect of the antibody on A431 cell proliferation was on the order of 35% (Figure 2A), in confirmation of previous results.[6] The down-regulation of VEGF production by C225 antibody is likely to operate at the level of gene transcription, given that treatment with 1 μg/ml C225 antibody resulted in a 50% decrease in expression of VEGF mRNA (Figure 2B).

Down-Regulation of VEGF Production and Inhibition of A431 Tumor Growth by C225 Antibody in Vivo

To further investigate the potential role of the C225 antibody as a possible anti-angiogenesis agent *in vivo*, we examined the effect of this antibody on the growth of established A431 tumors and their *in situ* VEGF production. Transient therapy consisting of only four injections of C225 antibody (spaced 2 days apart) into A431 tumor-bearing mice resulted in appreciable (treated to control ratio = 33%) inhibition of tumor growth (Figure 2C). As expected, staining for expression of proliferation-associated Ki67 antigen revealed a pronounced withdrawal of tumor cells from the cell cycle when the mice were exposed to C225 (Figure 3, E and F; Table 1). Such an effect may be either direct, as reported earlier, or secondary to inhibition of angiogenesis, as treatment of A431 tumors with anti-VEGF neutralizing antibody A4.6.1 also resulted in a decrease in numbers of Ki67-positive tumor cells, especially in the innermost part of the tumor (34% ± 4.6 positive cells in control *versus* 15% ± 3.1 in treated mice; $P = 0.0004$; data not shown). Therefore, it is possible that a similar anti-angiogenic effect would conceivably contribute to the therapeutic efficacy of C225 treatment, at least in the case of A431 tumors. Figure 3, A and B, shows the results of immunohistochemical staining for VEGF in C225-treated and control A431 tumor sections. Control tumors are highly positive for VEGF protein. This is particularly true for clusters of large tumor cells that are negative for Ki67, suggesting that the main source of VEGF in this case are nondividing rather than proliferating tumor cells. In contrast, tumors treated with C225 antibody are largely negative for VEGF staining with the exception of weakly positive clusters of the aforementioned large tumor cells. Consistent with this pattern, a twofold reduction in the average number of blood vessels in treated *versus* control tumors was also observed (Figure 3, C and D; Table 1). These *in vivo* results appear to corroborate those obtained *in vitro*.

Discussion

Many inhibitory signal transduction agents that disrupt the transforming functions of oncogenes and (proto)-

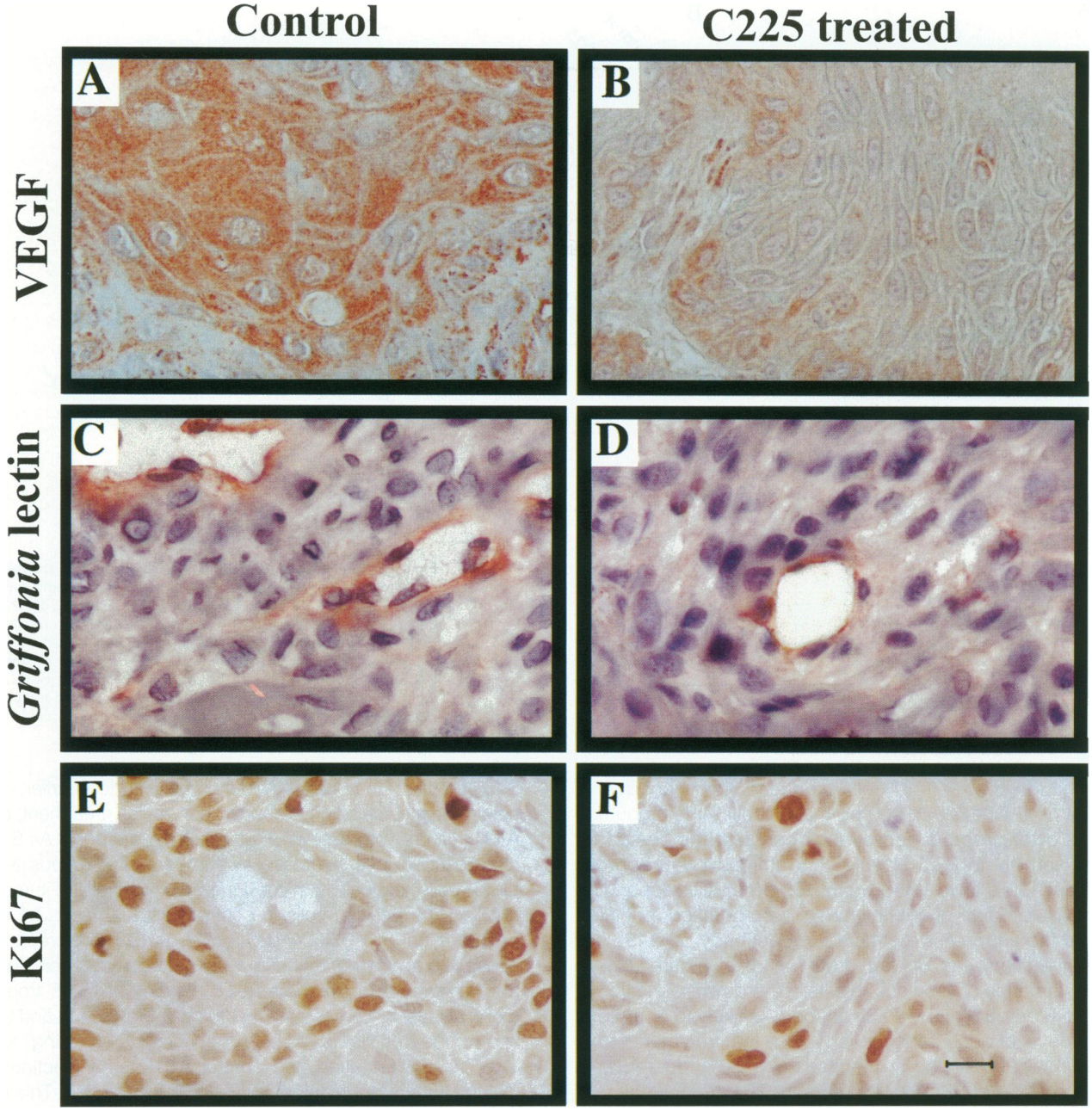

Figure 3. Immunohistochemical evaluation of VEGF expression and cellular proliferation in A431 tumors. A431 tumor-bearing mice were treated with anti-EGFR (C225) antibody (**B**, **D**, and **F**) or with PBS (**A**, **C**, and **E**). VEGF down-regulation is apparent in C225-treated A431 tumors (compare **A** and **B**). Blood vessel numbers were reduced in treated *versus* control mice (compare **C** and **D**). Consistent with an antiproliferative effect *in vivo*, Ki67 positivity was lower after C225 antibody treatment (compare **E** and **F**). Bar, 20 μm. See Table 1 and Materials and Methods for details.

oncogenes lack overt cytotoxic properties when tested against tumor cells in culture.[31,32] Nevertheless, they can bring about impressive cytotoxic-like effects against established solid tumors in various preclinical models.[5,6] These agents include the anti-EGFR and anti-ErbB2/*neu* monoclonal neutralizing antibodies used in the studies reported here.[6,7] Such a discrepancy can be explained, at least in part, by postulating that these agents may exert an anti-angiogenic effect *in vivo* by down-regulating one or more angiogenesis growth factors, including VEGF/VPF. Clearly, the therapeutic benefits of such an anti-angiogenic effect would not be apparent in cell-culture-based drug testing or screening protocols.

We chose to evaluate whether overexpression of the EGFR, and the EGFR-related receptor tyrosine kinase ErbB2/*neu*, stimulate VEGF/VPF production in solid tumors as various neutralizing monoclonal antibodies to the proteins encoded by these (proto)-oncogenes have been used extensively in preclinical therapeutic models and have now entered early-phase clinical trials.[4] Other methods of targetting proto-oncogene function in tumors are under active investigation, eg, gene therapy[33,34] and

Table 1. Immunohistochemical Analysis of Ki67, VEGF, and Blood Vessel Staining on Paraffin-Embedded and Frozen Sections from A-431 Tumors Grown on Nude Mice

Treatment	VEGF	Blood vessel counts ± SD	Ki67 (% ± SD)
Control (PBS)	(+++)	24.2 ± 4.7	30 ± 7.1 (6)
C225 MAb	(+)	10.3 ± 4.1	9 ± 3.7 (7)

VEGF cytoplasmic staining was assessed semiquantitatively by assigning a score based on color intensity produced by AEC red chromogen: −, negative; +, slightly positive; ++, moderately positive; +++, highly positive.

Percentages of positive cells and blood vessel counts were determined on a minimum of 10 high-power (40×) fields (100 cells/field in the case of Ki67) per slide and two slides per sample. Two-tailed *P* value was <0.0001 in both cases. See Materials and Methods for details. Numbers of tumors evaluated are indicated in parentheses.

synthetic receptor antagonists.[35] Our results, considered as a whole, strongly implicate the EGFR and ErbB2/*neu* as inducers of VEGF/VPF and, hence, by extension, tumor angiogenesis. For example, in the case of EGFR, treatment of the human epidermoid carcinoma A431 cell line with the C225 monoclonal anti-EGFR antibody resulted in a dose-dependent inhibition of VEGF/VPF expression at both the mRNA and protein levels. Significantly, this decline in VEGF/VPF was not restricted to *in vitro* treatments; four injections of C225 antibody into nude mice with an established A431 human epidermoid carcinoma xenograft resulted in an obvious down-regulation of VEGF/VPF expression, which accompanied the tumor growth inhibition. Similar conclusions were reached in the context of ErbB2/*neu*. Thus, transfection of a transforming-competent, mutant ErbB2/*neu* oncogene into mouse NIH 3T3 cells resulted in a dramatic up-regulation of VEGF/VPF expression, and neutralizing antibodies to human ErbB2/*neu* could cause a detectable suppression of VEGF/VPF expression in SKBR-3 breast cancer cells, which are known to be VEGF/VPF -positive and overexpress ErbB2/*neu*. These results may be pertinent to the prognostic link recently established between tumor angiogenesis and c-erbB2/*neu* expression in nasopharyngeal cancer.[36]

One obvious question our findings raise is whether it is reasonable to propose that an anti-tumor effect could result, at least in part, by inhibition of angiogenesis when the degree of VEGF/VPF suppression induced by the antibody treatments is in the range of twofold *in vitro* and perhaps slightly more *in vivo*. For several reasons, we feel the answer is yes. First, studies in VEGF knockout mice have shown that disruption of only a single VEGF allele, equivalent to 50% reduction of VEGF protein, is sufficient to block vasculogenesis and angiogenesis to such an extent that embryos die between days 11 and 12 gestation.[16] This remains an unprecedented finding. Second, induced suppression of VEGF protein expression in a human glioblastoma by only threefold, assessed by using antisense genetic methods, can almost completely obliterate the tumorigenic ability of such variant cells in nude mice.[17] Third, it is clearly probable that the antibody treatments we studied would suppress the expression of some additional growth factors known to be pro-angiogenic, eg, basic fibroblast growth factor, interleukin-8, TGF-α, and TGF-β, as shown by Ciardiello et al.[37] This would clearly enhance the potential anti-angiogenic activity of the antibody treatments. Nevertheless, in the case of A431 squamous carcinoma cells, it has already been shown that their growth in mice can be almost completely blocked by inhibiting the function of the flk-1 (VEGFR2) mouse endothelial cell receptor for VEGF.[18] Thus, even if the EGFR antibody treatment inhibited VEGF expression *in vivo* in A431 cells by approximately 50%, and did not suppress any other angiogenic growth factor, it would still be reasonable to postulate that an anti-angiogenic effect could ensue from such a treatment. It is therefore not surprising that we also found a significant reduction in the extent of tumor vascularity in the A431 tumors removed from nude mice after only four treatments with the C225 antibody spaced 2 days apart, a finding consistent with our anti-angiogenesis hypothesis. The extent of reduction in blood vessel counts, in the range of twofold, is in line with the results of others, eg, Cheng et al,[17] who observed a threefold reduction in such counts in tumors obtained from injection of VEGF antisense transfected cells where the growth of such tumors was profoundly suppressed *in vivo*.

Finally, our results could be very important to the issue of the therapeutic index that can be attained by anti-tumor agents such as anti-EGFR or anti-ErbB2/*neu* neutralizing antibodies. This is because, unlike cell proliferation, angiogenesis is not normally a prominent physiological process in healthy humans, with the exceptions of corpus luteum development in females. Thus, if the effect of such anti-tumor agents is mediated through suppression of tumor angiogenesis in addition to inhibition of tumor cell growth, the therapeutic index would be increased. This could help explain why these agents can exert anti-tumor effects *in vivo* that seem out of proportion with their generally modest anti-tumor effects in monolayer cell culture. It should be noted that such an *in vitro/in vivo* discrepancy may also be due to a pro-apoptotic effect mediated by these anti-tumor agents on tumor cells growing in a multicellular and/or anchorage-independent context, as opposed to monolayer context. Indeed, genetic or pharmacological disruption of mutant *ras* genes or RAS proteins can lead to a high degree of apoptosis of mutant *ras*-transformed cells growing in three-dimensional culture[38] or anchorage independently[39] but not in monolayer cell culture, where only an anti-proliferative effect is observed.

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